Supplemental Material

Pacific Ocean–wide Profile of CYP1A1 Expression, Stable Carbon and Nitrogen Isotope Ratios, and Organic Contaminant Burden in Sperm Whale Skin Biopsies

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Research permits: Samples were obtained under U.S. National Marine Fisheries Service permit No. 1004 to Ocean Alliance and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) permit 00US19824/9; Mexico Secretaria de Medio Ambiente Recursos Naturales y Pesca permit No. 4903 to Dr. Jorge Urban Ramirez of the Universidad Autonoma de Baja California Sur, Mexico; Galapagos Parque Nacional Galapagos de Instituto Ecuatoriano Forestal y de areas Naturales permit No. 735-93 SPNG/GL to Dr. Jorge Reynolds of the Whale Heart Satellite Tracking Program (Colombia); Kiribati permit issued by Tooti Tekinaiti, Ministry of Natural Resource Development Fisheries Division Ref. FDG: 1/46; and the Papua New Guinea permit issued through collaboration between the Papua New Guinea's Marine Scientific Research sub-Committee, Department of Foreign Affairs and the Department of Environment and Conservation.

Immunohistochemistry (IHC): We prepared samples for CYP1A1 immunohistochemistry with the monoclonal antibody MAb 1-12-3, as previously described (Godard et al. 2004). MAb 1-12-3 is specific for CYP1A1 in mammals (Drahushuk et al. 1998) and has been shown to identify a single band in beluga whale (*Delphinapterus leucas*) liver microsomes after Western blotting (White et al. 1994) and a single protein in human liver microsomes following 2D electrophoresis (Drahushuk et al. 1998). We assessed CYP1A1 staining under light microscopy after incubation with amino-9-ethylcarbazole as chromogenic substrate (AEC, Signet Laboratories) and counterstaining with Mayer's hematoxylin (Sigma). CYP1A1 expression was quantified as a staining score (0-15) calculated as the product of staining occurrence (0-3) and intensity (0-5) (Godard et al. 2004). This scoring technique has been found to accurately reflect CYP1A1 protein expression (Woodin et al. 1997). Slides were scored blind and in random order. In addition, we estimated proxy CYP1A1 IHC scores for pooled samples from each region by

summing individual sample IHC scores multiplied by the contribution of the individual section to the overall pooled sample weight. We evaluated tissue integrity (nuclear stain intensity, nucleus shape, eosinophilia) using hematoxylin- and eosin-treated slides to confirm that samples were adequate for IHC.

Analytical chemistry

-*Individual samples:* We selected 10 samples from each region for individual sample assays, including four samples selected at random from samples within the highest (upper 33%) CYP1A1 IHC score group for each region, and three each from the median and lower third score groups for each region. These samples were analyzed for polychlorinated biphenyl (PCB), dichlorodiphenyltrichloroethane (DDT), and hexachlorobenzene (HCB) according to U.S. EPA Method 8081/8082 as previously described (Ballschmiter and Zell 1980) with modification (Marsili and Focardi 1996). We calculated percentage of extracted organic material (%EOM) for each subsample after lyophilization and extraction with n-hexane. Small subsample sizes prevented further determination of lipid content in the extracted organic material. We spiked each sample prior to extraction with 2,4,6-trichlorobiphenyl (PCB 30) to calculate recovery. Sample extracts were treated with sulphuric acid and then underwent Florisil column chromatography to further purify the lipid phase. We used PCB 209 and a mixture of Aroclor 1260, hexachlorobenzene and pp'- and op'-DDT, DDD and DDE as internal and calibration standards respectively. Capillary gas chromatography was performed on an SBP-5 column (30m x 0.2mm i.d.) with electron capture detection. The carrier gas was N_2. Make-up gas was argon/methane (95/5). Thirty congeners were resolved (Supplemental Material, Table 1) using a temperature gradient. Total PCB concentrations (ΣPCBs) were quantified as the sum of all congeners analyzed. Sample size precluded separate analysis of dioxin-like PCBs (two mono-

ortho-substituted PCBs, 118 and 156, were detected but in conjunction with non dioxin-like congeners). Total DDT concentrations (ΣDDTs) were calculated as the sum of *op'*DDT, *pp'*DDT, *op'*DDD, *pp'*DDD, *op'*DDE and *pp'*DDE. Results were expressed in ng/g EOM.

-*Pooled samples*: We pooled all samples collected in each region by sex (eight pooled samples total since we collected only males in GP and females in KR) to obtain enough material to determine polycyclic aromatic hydrocarbons. We prepared pooled samples by combining complete vertical sections of blubber samples (section weight range = 3.7-184 mg). Small sample sizes prevented pooling of sections of identical weight. PAHs were extracted according to Griest and Caton (1983) with modifications (Marsili et al. 2001) and analyzed by HPLC with fluorescence detection as described in Marsili et al. (2001). The external standard consisted of sixteen PAHs from Supelco (EPA 610 PAH mixture). Results were expressed as the sum of fifteen PAHs (ΣPAHs: naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene, indeno(1,2,3-cd)pyrene) per ng/g EOM. Recoveries ranged from 80-98%; no PAHs were detected in blanks. We also analyzed ΣPCBs, ΣDDTs, HCB, and %EOM in each set of pooled samples in relation to proxy CYP1A1 IHC scores for the pooled samples. Separate analysis of dioxin-like PCBs was precluded by small sample size.

Stable isotope analyses: 100 skin subsamples were dried and ground into a fine powder using liquid nitrogen and a mortar and pestle. Subsamples were randomly selected amongst animals biopsied at locations where fish or squid were concurrently collected. We analyzed 1 mg of each powdered subsample using a Carlo-Erba 1108 elemental analyzer coupled to a Thermo Finnigan Delta-S isotope ratio mass spectrometer. Stable carbon and nitrogen isotope ratios are expressed

as $\delta^{13}C$ or $\delta^{15}N$ (‰) = $[(R_{sample}/R_{standard}) - 1] \times 10^3$, where R is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ for $\delta^{13}C$ or $\delta^{15}N$, respectively. Laboratory standards were referenced to Pee Dee Belemnite for carbon and atmospheric air for nitrogen. Standard deviations for repeated measurements of yeast standard were 0.2‰.

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Supplemental Material, Table 1. Nomenclature and structure of PCB congeners present in analyzed samples according to IUPAC (Ballschmiter and Zell 1980)

IUPAC number	Structure	IUPAC number	Structure
Pentachlorobiphenyls		Heptachlorobiphenyls	
PCB 95	2,2',3,5',6	PCB 170	2,2',3,3',4,4',5
PCB 99	2,2',4,4',5	PCB171	2,2',3,3',4,4',6
PCB 101	2,2',4,5,5'	PCB172	2,2',3,3',4,5,5'
PCB 118	2,3',4,4',5	PCB174	2,2',3,3',4,5,6'
Hexachlorobiphenyls		PCB177	2,2',3,3',4',5,6
PCB 128	2,2',3,3',4,4'	PCB178	2,2',3,3',5,5',6
PCB 135	2,2',3,3',5,6'	PCB180	2,2',3,4,4',5,5'
PCB 138	2,2',3,4,4',5'	PCB183	2,2',3,4,4',5',6
PCB 141	2,2',3,4,5,5'	PCB187	2,2',3,4',5,5',6
PCB 144	2,2',3,4,5',6	Octachlorobiphenyls	
PCB 146	2,2',3,4',5,5'	PCB194	2,2',3,3',4,4,'5,5'
PCB 149	2,2',3,4',5',6	PCB 195	2,2',3,3',4,4',5,6
PCB 151	2,2',3,5,5',6	PCB 196	2,2',3,3',4,4',5',6
PCB 153	2,2',4,4',5,5'	PCB199	2,2',3,3',4',5,5',6'
PCB 156	2,3,3',4,4',5	PCB201	2,2',3,3',4,5',6,6'
		PCB202	2,2',3,3',5,5',6,6'
		Nonachlorobiphenyls	
		PCB206	2,2',3,3',4,4',5,5',6

All listed PCBs were analyzed individually except for the following combined sets: (PCBs 144 and 135), (PCBs 149 and 118), and (PCBs 156, 171 and 202).

Supplemental Material, Table 2. CYP1A1 protein expression in skin endothelial cells, smooth muscle cells, and fibroblasts of sperm whales from five Pacific Ocean collection sites. [a, b]

Region	Endothelial Cells			Smooth Muscle Cells			Fibroblasts		
	All Animals	Males	Females	All Animals	Males	Females	All Animals	Males	Females
Gulf of California (78)	0.8 ± 0.1 (0-3)	0.8 ± 0.1 (0-2)	0.8 ± 0.1 (0-3)	1.3 ± 0.1 (0-4)	1.2 ± 0.2 (0-3)	1.3 ± 0.1 (0-4)	0.7 ± 0.1 (0-3)	0.8 ± 0.1 (0-2)	0.7 ± 0.1 (0-3)
Galapagos (25)	2.1 ± 0.4 (0-7.5)	2.1 ± 0.4 (0-7.5)	-	2.6± 0.4 (0-7.5)	2.6± 0.4 (0-7.5)	-	1.8 ± 0.2 (0-4)	1.8 ± 0.2 (0-4)	
Pacific Crosing (34)	0.3 ± 0.0 (0-1)	0.3 ± 0.1 (0-0.5)	0.3 ± 0.1 (0-1)	0.5 ± 0.1 (0-1.5)	0.5 ± 0.1 (0-1)	0.5 ± 0.1 (0-1.5)	0.3 ± 0.1 (0-1)	0.3 ± 0.1 (0-1)	0.3 ± 0.1 (0-1)
Kiribati (17)	0.5 ± 0.1 (0-1)	-	0.5 ± 0.1 (0-1)	0.6 ± 0.1 (0-1.5)	-	0.6 ± 0.1 (0-1.5)	0.4 ± 0.1 (0-1)	-	0.4 ± 0.1 (0-1)
Papua New Guinea (80)	0.6 ± 0.1 (0-2)	0.5 ± 0.2 (0-2)	0.6 ± 0.1 (0-2)	0.8 ± 0.1 (0-3)	0.8 ± 0.2 (0-2)	0.8 ± 0.1 (0-3)	0.5 ± 0.1 (0-2)	0.3 ± 0.1 (0-0.5)	0.5 ± 0.1 (0-2)
All sites (234)	0.8 ± 0.1 (0-7.5)	1.1 ± 0.2 (0-7.5)	0.6 ± 0.0 (0-3)	1.1 ± 0.1 (0-7.5)	1.5 ± 0.2 (0-7.5)	0.9 ± 0.1 (0-4)	0.7 ± 0.0 (0-4)	0.9 ± 0.1 (0-4)	0.6 ± 0.0 (0-3)

[a]CYP1A1 expression levels are staining score means ± SEM (range). [b]Sample size is shown next to region name and includes all male and female animals analyzed.

Supplemental Material, Table 3. Spearman Rank correlations among %EOM and ΣPCB, ΣDDT, and HCB burden in individual samples.

Individual samples	ΣPCBs	ΣDDTs	HCB
%EOM	*-0.4667	*-0.3397	*-0.4234
ΣPCBs		*0.5023	*0.3126
ΣDDTs			-0.0999

* indicates statistical significance at $p<0.05$.

Supplemental Material, Table 4. Spearman Rank correlations between IHC scores, %EOM, and contaminant burdens in individual blubber samples. None but one correlation coefficient were statistically significant.

All animals (n=50)	%EOM	ΣPCBs	ΣDDTs	HCB
Endothelial CYP1A1 IHC Score	0.0389	0.1192	0.0153	0.1625
Smooth muscle CYP1A1 IHC Score	-0.1457	0.2687	0.0694	0.1274
Fibroblast CYP1A1 IHC Score	-0.0755	0.1342	0.2193	0.1064
Males only (n=17)				
Endothelial CYP1A1 IHC Score	-0.0975	0.1521	0.2152	*0.6070
Smooth muscle CYP1A1 IHC Score	-0.2423	0.3835	0.2261	0.2958
Fibroblast CYP1A1 IHC Score	-0.0614	0.4286	0.4675	0.3212
Females only (n=33)				
Endothelial CYP1A1 IHC Score	0.0949	0.1885	-0.1208	0.0755
Smooth muscle CYP1A1 IHC Score	-0.0628	0.3098	-0.0817	0.1396
Fibroblast CYP1A1 IHC Score	0.0109	0.0365	-0.0563	0.1018

* indicates statistical significance at $p<0.05$.

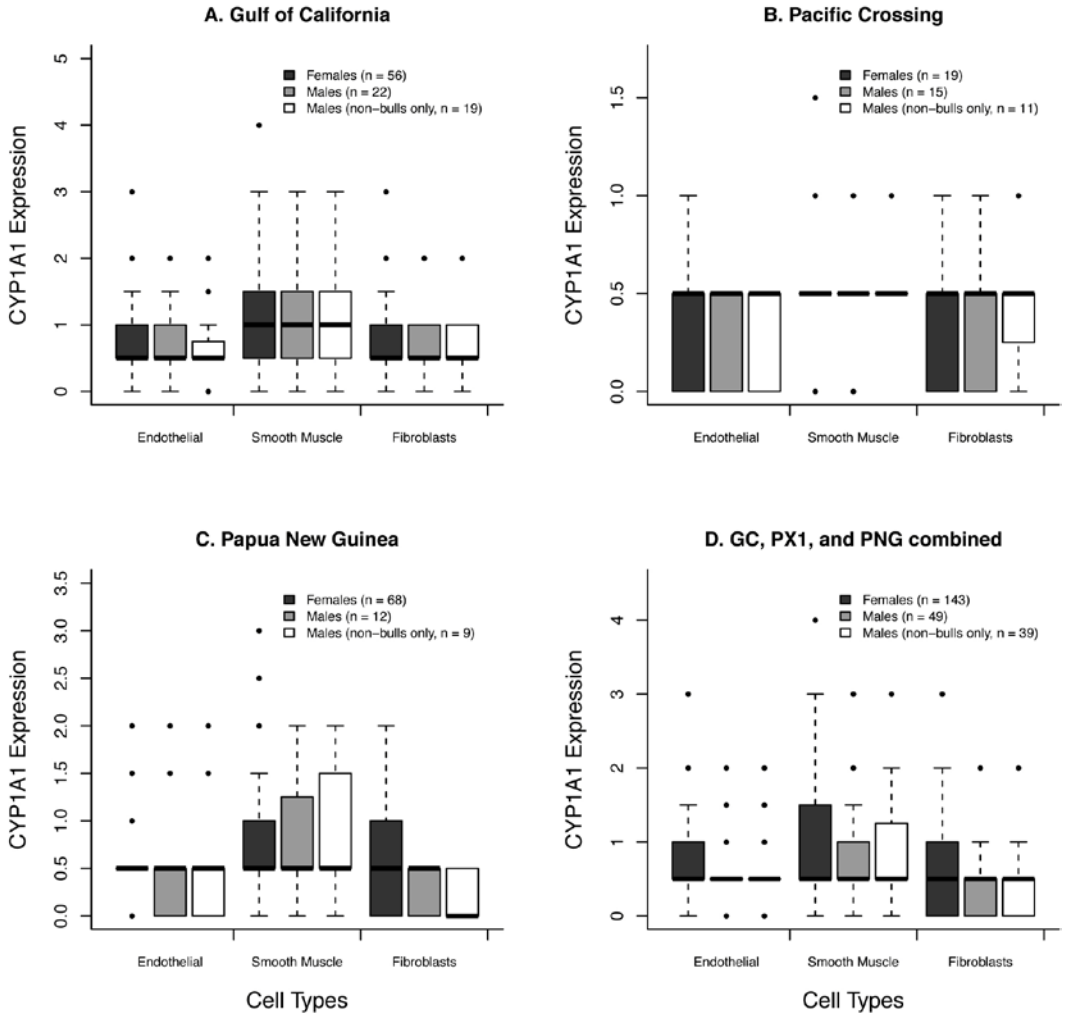
Supplemental Material, Table 5. Spearman Rank correlations between proxy IHC scores, %EOM, ΣPCBs, and ΣPAHs in pooled samples. None of the correlation coefficients were statistically significant.

All pooled samples (n=8)	%EOM	ΣPCBs	ΣPAHs
Proxy endothelial CYP1A1 IHC Score	0.6190	-0.2619	0.40478
Smooth muscle CYP1A1 IHC Score	0.3095	-0.2380	0.28578
Fibroblast CYP1A1 IHC Score	0.1190	-0.2142	-0.0238
Male pooled samples only (n=4)			
Endothelial CYP1A1 IHC Score	0.200	-0.800	0.800
Smooth muscle CYP1A1 IHC Score	-0.400	-1.000	0.600
Fibroblast CYP1A1 IHC Score	-0.600	-0.400	-0.400
Females pooled samples only (n=4)			
Endothelial CYP1A1 IHC Score	1.000	-0.800	0.600
Smooth muscle CYP1A1 IHC Score	1.000	-0.800	0.600
Fibroblast CYP1A1 IHC Score	0.800	-0.600	0.800

Supplemental Material, Table 6: Comparison of sperm whale skin nitrogen (N) and carbon (C) stable isotope ratios among regions.[a]

Collection Region	All Animals		Males		Females	
Sample Size (all, males, females)	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$
Gulf of California	17.8 ± 0.1 ^{x}	-16.8 ± 0.1 ^{xyz}	17.7 ± 0.2 ^{x}	-16.7 ± 0. 2 ^{x}	17.8 ± 0.1 ^{x}	-16.8 ± 0.1 ^{x}
(39, 13, 25)	(16.5 to 18.9)	(-18.1 to -15.6)	(16.8 to 18.6)	(-17.5 to -15.9)	(16.5 to 18.9)	(-17.8 to -15.6)
Galapagos Island	14.3 ± 0.2 ^{y}	-17.3 ± 0.1 ^{zht}	14.3 ± 0.2 ^{y}	-17.3 ± 0.1 ^{xy}	-	-
(12, 12, 0)	(13.3 to 16.0)	(-18.1 to -16.6)	(13.3 to 16.0)	(-18.1 to -16.6)	-	-
Pacific Crossing1	15.0 ± 0.3 ^{y}	-17.5 ± 0.1 ^{ht}	15.0 ± 0.4 ^{y}	-17.7 ± 0.2 ^{x}	14.9 ± 0.3 ^{y}	-17.4 ± 0.1 ^{x}
(19, 8, 11)	(12.6 to 16.8)	(-18.7 to -16.8)	(13.2 to 16.7)	(-18.7 to -16.9)	(12.6 to 16.8)	(-18.0 to -16.8)
Kiribati	15.1 ± 0.1 ^{y}	-17.1 ± 0.1 ^{xt}	-	-	15.1 ± 0.1 ^{y}	-17.1 ± 0.1 ^{x}
(8, 0, 8)	(14.6 to 15.4)	(-17.4 to -16.6)	-	-	(14.6 to 15.4)	(-17.4 to -16.6)
Papua New Guinea	14.7 ± 0.1 ^{y}	-16.2 ± 0.2 ^{y}	14.1	-15.8	14.7 ± 0.1 ^{y}	-16.2 ± 0.2 ^{y}
(22, 1, 21)	(14.0 to 15.7)	(-19.1 to -14.5)	-	-	(14.0 to 15.7)	(-19.1 to -14.5)

[a]Stable isotope ratios are means ± SEM (range). Different letters indicate statistically different means ($p<0.05$) as determined by Tukey-Kramer's HSD for each stable isotope and within each category (all, males, females). In males, PNG data were not included in the analyses of means since n=1. One GC sample could not be sex-typed.



Supplemental Material, Figure 1. Lack of sex effect on CYP1A1 expression in endothelial cells, smooth muscle cells and fibroblasts in sperm whale skin biopsies. CYP1A1 expression in animals biopsied in the Gulf of California (Panel A), Pacific Crossing sampling region (Panel B), Papua New Guinea (Panel C), and all three regions combined (Panel D). Note: Animals sampled either in the Galapagos or the Kiribati survey regions were of only one sex and therefore were not included. Expression levels are presented as staining score means for each sex and cell type. Error bars represent the standard error of the mean. Staining scores in females did not differ statistically from those of males (bulls and non-bull males) or non-bull males only ($p<0.05$). The non normality of CYP1A1 scores and the small numbers of bulls precluded comparisons between the two male categories.